

Original article

The time course of the bilateral release of cytokines and mediators after unilateral nasal allergen challenge

Background: Late phase reactions after allergen challenge can be understood as a correlate of the inflammatory reaction in allergic rhinitis.

Methods: To investigate which cytokines are involved in it and to dissect direct and indirect effects of nasal allergen challenge, we performed unilateral nasal allergen provocation with the disc method in 12 seasonal allergic volunteers. Symptom scores, nasal secretions and nasal airflow were quantified. In the secretions that were collected in the early phase and for 8 h after provocation, we measured histamine, and the cytokines interleukin (IL)-1 β , IL-8, IL-4, and the natural antagonist of IL-1 β , IL-1 receptor type 1 (IL-1Ra) using enzyme-linked immunosorbent (ELISA)-assays. Control challenges with diluent instead of allergen were performed in all subjects.

Results: We demonstrated a bilateral increase in nasal secretion weights in the early and late phase. Histamine was significantly increased in the early and late phase in nasal secretions from both nostrils. IL-1 β increased in the late phase only, where it was also found on the unchallenged, contralateral side. Its antagonist IL-1Ra was found in very high quantities (1000-fold higher than IL-1 β) but demonstrated only marginal changes after provocation. IL-8 was increased in both nostrils early and late after challenge, whereas IL-4 was significantly elevated in the late phase.

Conclusions: We described the time course of mediator and cytokine release into nasal secretions after allergen challenge. We hypothesize that the observed indirect effects on the unchallenged, contralateral side can be at least partially attributed to neuronal reflexes.

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Key words: allergic rhinitis; cytokine antagonists; cytokines; late phase reaction; neuronal inflammation.

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Accepted for publication 24 February 2005

Nasal allergen provocation has proven to be an indispensable tool to investigate the pathophysiology of allergic rhinitis. This technique allowed to differentiate between the early and late phase of the allergic reaction in the nose (1) and to describe the time course of release for several inflammatory mediators (2). To investigate both sides of the nose independently, Baroody et al. established a technique of unilateral nasal challenge with the concomitant bilateral collection of nasal secretions using filter paper discs (the disc method) (3). Utilizing this modification, nasomaxillary reflexes could be demonstrated after a unilateral nasal allergen challenge with histamine (4).

The late phase exhibits many features of the natural disease such as the influx of inflammatory cells into the mucosa. There is a lot of support for the notion that most aspects of the allergic inflammation are controlled by cytokines. Especially Th2-cytokines could be linked in many different experimental settings to the characteristic changes found in allergic rhinitis. Therefore, we per-

formed this study to determine the time course of the release of proinflammatory and Th2-cytokines in nasal secretions after allergen challenge and additionally to investigate contralateral effects after a unilateral challenge in seasonal allergic volunteers.

The choice of the specific cytokines in our study was based on the following considerations, we chose interleukin (IL)-1 β for two reasons: first, it is a typical member of the group of proinflammatory cytokines; secondly, we were interested to compare the release of this cytokine to its naturally occurring antagonist, IL-1 receptor type 1 (IL-1Ra). The IL-4 was chosen because of its cardinal role in the regulation of the allergic phenotype. We hypothesized that it is released in the late phase of the allergic reaction. The IL-8 was selected as a characteristic member of the group of C-X-C chemokines and because it has been demonstrated to be released by mast cells after immunoglobulin E (IgE) cross-linking. As we and others demonstrated that unilateral nasal allergen challenge

induces a nasosnasal reflex with contralateral effects on physiological parameters and the contralateral release of mediators (3, 5), an additional aim of our study was to find out if these cytokines can also be found on the unchallenged side of the nose.

Material and methods

Subjects and design

Twelve seasonal allergic volunteers (ages 21–29 years), sensitized to grass, birch or hazelnut pollen participated in the study. They were defined as seasonal allergies on the basis of history, skin prick tests, specific IgE-levels and nasal allergen provocation. All subjects were challenged out of the allergen season and had not taken any medication except oral contraceptives within the last month. They were intranasally challenged with the appropriate allergen using filter paper discs and followed for 8 h. Separated by at least 2 weeks, control challenges with the diluent alone were carried out in all participants. The Ethics Committee of the Heinrich-Heine-University approved the trial and each subject gave written informed consent before entering the study.

Localized nasal challenge

Filter paper discs (10 mm in diameter, 1.2 mm thickness, punched out from Shandon filter cards; Shandon Inc., Pittsburgh, PA, USA) were used for nasal allergen challenges and for the collection of nasal secretions (6). The discs were soaked with 75 µl of challenge solutions and applied to the middle portion of the anterior nasal septum, posterior to the mucocutaneous junction for 1 min. Five minutes after their removal and from then on for every hour, nasal secretions were collected from the same site and from the corresponding area on the contralateral nasal septum using dry, pre-weighed discs left on the mucosa for 45 s. After collection of nasal secretions, the discs were immediately placed into a 5 ml tube and this combination was weighed allowing the calculation of the amount of generated secretions by comparison with the weight of the tube/disc combination before challenge. Each collection disc was eluted in 1250 µl of 0.9% sodium chloride solution for 3 h at 4°C. The disc was then removed and the eluate stored in 250 µl aliquots at –80°C until assayed.

Cytokine and histamine assays

Histamine, IL-1β, IL-1Ra, IL-4 and IL-8 in nasal secretions were measured using commercially available enzyme-linked immunosorbent (ELISA)-assays (R&D Systems, Wiesbaden, Germany; histamine: Immunotech, Krefeld, Germany). The sensitivities of the assays were: histamine: 22.2 ng/ml, IL-1β: 0.083 pg/ml, IL-1Ra: 31.2 pg/ml, IL-4: 0.13 pg/ml, IL-8: 31.2 pg/ml. We performed control experiments by spiking discs with a solution containing all cytokines and histamine (dissolved in unstimulated nasal lavage fluid) to test for reproducibility, recovery rates and cross-reactivity. The reproducibility was found to be satisfying (< 5% difference in repeated measurements). Recovery rates for the cytokines ranged from 50 to 80% and remained very constant for the individual cytokines. No cross-reactivity between the different cytokines could be found. The histamine and cytokine values presented here are the total amounts recovered by the individual collection discs. These values were calculated by multiplying the measured concentration

by the volume of the collected secretion plus the volume of the eluate (1250 µl).

Allergen solutions

Mixed grass, birch or hazelnut allergen extracts (Allergopharma, Reinbek, Germany) in a concentration of 33.333 BU/ml were used. Each challenge disc therefore contained 2.500 BU of allergen. To control for nonspecific irritation, challenges with the diluent alone (sterile phenol-buffered saline; Allergopharma) were performed before allergen challenges.

Challenge protocol

Before the challenge procedure anterior rhinoscopy was performed to confirm the absence of nasal abnormalities. At all time-points, a symptom score was obtained using a visual analogue scale (three separate scores for secretion, obstruction, irritation; for the analysis they were added together; possible value ranges from 0 to 30), sneezes were counted and nasal airway flow was measured bilaterally using anterior rhinomanometry (recorded at 150 Pa with Atmos Rhinomanometer 300, Atmos, Germany). Subsequently, the first two pairs of collection discs, separated by five consecutive nasal lavages with 5 ml of physiological saline solution (37°C) in each nostril (to bring levels of pre-existing mediators to baseline), and a 5-min interval, were applied to collect baseline secretions and mediators. These were followed by the first diluent challenge disc applied unilaterally. About 5 min after removal, secretions were collected from the same site of challenge and the corresponding area on the contralateral septum. Then challenge discs with allergen were applied followed by collection discs after 5 min. Thereafter, collection discs were applied hourly for 8 h. During the control challenge identical measurements were performed and the allergen was substituted by diluent.

Statistical methods

All data are presented as median and 25–75 percentiles. Because the data were not normally distributed, nonparametric statistics were employed to compare the responses of diluent and allergen challenge. Friedman analysis of variance was used to examine differences between points on the same curve. If a significant overall difference was found, *post hoc* analysis was performed using Wilcoxon matched pairs signed rank test to compare different points within the same curve. Unless otherwise noted, results from Wilcoxon tests are given. Two-tailed *P*-values are reported, *P* < 0.05 is considered statistically significant.

Results

Symptoms

Nasal allergen challenge lead to a significant increase in symptom scores immediately after provocation [diluent: 0.45 (0–3.50); allergen (5 min): 21.75 (11.75–25.25)]. In the following hours the levels decreased slowly, remaining significantly elevated until the sixth hour [4.35 (1.75–10.75)].

The number of sneezes was increased in the minutes after the allergen challenge [diluent: 0 (0–0); allergen (5 min): 5.50 (4.0–10.0)] returning to zero thereafter.

Rhinomanometry

Nasal flow decreased significantly from 304 (195–334) cm³/s (diluent) to 132 (101–182) cm³/s 5 min after allergen challenge and remained significantly lowered at 1 [240 (112–263) cm³/s], 2 [204 (77–219) cm³/s] and 6 h [192 (109–310) cm³/s] on the ipsilateral side. On the contralateral side its drop immediately after the allergen provocation was less pronounced [128 (95–286) cm³/s] although still statistically significant. In the later hours there was no significant difference on this side.

Secretion weights

Nasal secretion weights significantly increased on both the ipsilateral and the unchallenged side, reaching their highest values 5 min after allergen provocation [ipsilateral: 73.5 (59.5–83.0) mg; contralateral: 59.5 (34.5–76.5) mg] and slowly decreased for the following hours (Fig. 1). The secretion weights were lower on the contralateral side.

Histamine

Histamine was released into nasal secretions on both sides after unilateral allergen challenge. Its peak occurred 5 min after allergen [ipsilateral: 8.8 (3.1–20.7) ng, contralateral: 1.1 (0.4–3.6) ng], then the levels decreased at 1 h and continued to rise again, reaching a second peak at the fifth hour on the ipsilateral [4.3 (1.8–7.0) ng] and at the sixth hour on the contralateral side [2.0 (0.5–4.9) ng; Fig. 2].

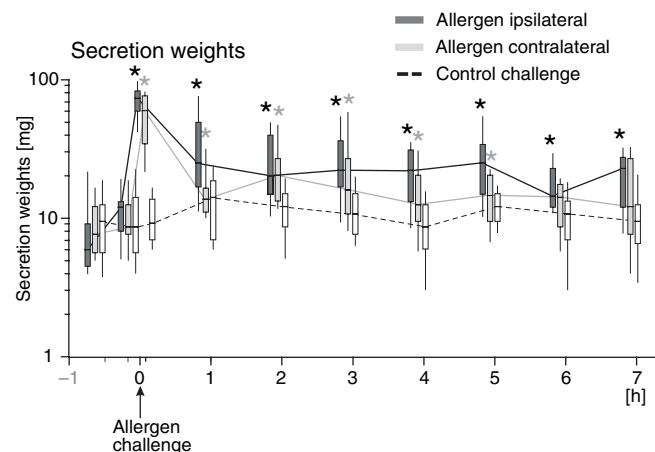


Figure 1. Time course of bilateral nasal secretion weights measured with the disc method after unilateral nasal allergen challenge. The arrow indicates the time of the allergen challenge. The dashed line represents values obtained after control challenge on a separate day using the same protocol [logarithmic y-axis, median and percentiles (10, 25, 50, 75, 90), $n = 12$, $*P < 0.05$, Wilcoxon-test].

Interleukin-1 β

The time course for IL-1 β in ipsilateral nasal secretions was different as it did not significantly increase before the second hour after allergen challenge [2.8 (1.9–3.1) pg] and continued to rise until the fifth hour [8.0 (0.7–11.6) pg; Fig. 3]. The run of the curve was similar on the contralateral side, but only at 6 h [2.2 (1.3–4.6) pg] its values were significantly elevated.

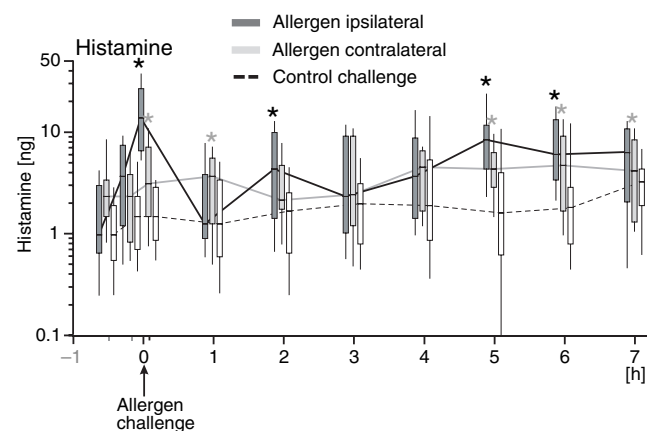


Figure 2. Time course of histamine content in bilateral nasal secretions after unilateral nasal allergen challenge. The arrow indicates the time of the allergen challenge. The dashed line represents values obtained after control challenge on a separate day using the same protocol [logarithmic y-axis, median and percentiles (10, 25, 50, 75, 90), $n = 12$, $*P < 0.05$, Wilcoxon-test].

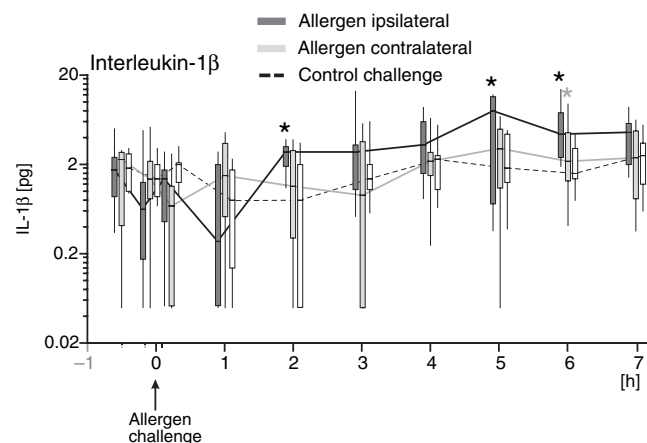


Figure 3. Time course of interleukin-1 β content in bilateral nasal secretions after unilateral nasal allergen challenge. The arrow indicates the time of the allergen challenge. The dashed line represents values obtained after control challenge on a separate day using the same protocol [logarithmic y-axis, median and percentiles (10, 25, 50, 75, 90), $n = 12$, $*P < 0.05$, Wilcoxon-test].

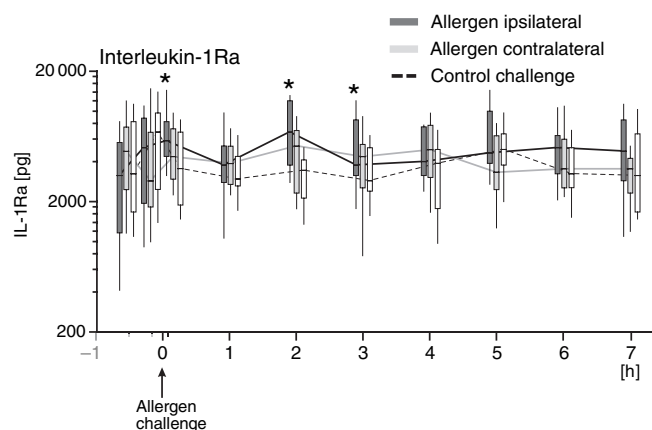


Figure 4. Time course of interleukin-1Ra content in bilateral nasal secretions after unilateral nasal allergen challenge. The arrow indicates the time of the allergen challenge. The dashed line represents values obtained after control challenge on a separate day using the same protocol [logarithmic y-axis, mean $\pm$ SEM, $n = 12$, $*P < 0.05$, Wilcoxon-test].

Interleukin-1Ra

The IL-1Ra was found in very high amounts in nasal secretions and demonstrated a modest increase on the ipsilateral side only. It was significantly elevated compared with diluent challenge [5216 (1914–8729) pg], 5 min [5933 (4416–8275) pg], 2 h and 3 h after allergen provocation (Fig. 4).

Interleukin-8

Interleukin-8 increased on both sides after unilateral nasal allergen provocation. Its curve had two peaks: one 5 min after allergen [ipsilateral: 139.5 (71.6–198.7) pg; contralateral: 126.3 (91.9–166.5) pg] and the second 5 h later on the ipsilateral side [281.6 (52.2–763.2) pg] and 4 h later in the contralateral nostril [203.0 (83.7–247.4) pg; Fig. 5].

Interleukin-4

The IL-4 was found in relatively low amounts in nasal secretions but was significantly increased 4, 5 [0.28 (0.16–1.46) pg] and 7 h after provocation on the ipsilateral side (Fig. 6). On the contralateral side, IL-4 was significantly elevated over diluent challenge [0.16 (0.16–0.16) pg] at the 5 min [0.17 (0.16–0.43) pg], 2 h and 3 h time-points.

No statistically significant changes for any of the parameters were observed after control challenge with the diluent alone and follow up for the same period.

Discussion

This study confirms the notion that allergic rhinitis is an inflammatory reaction that is not confined to the early

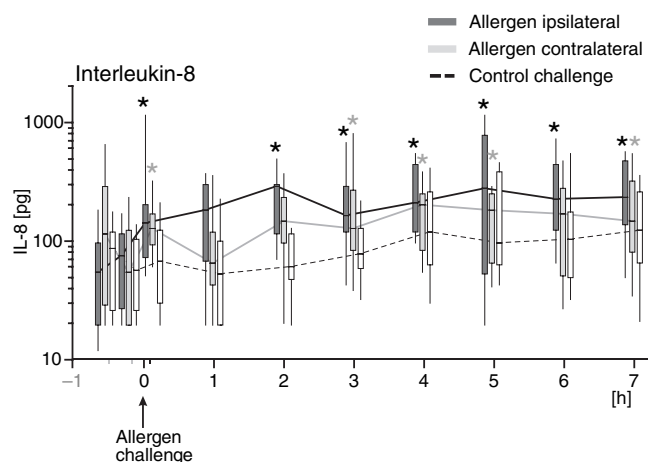


Figure 5. Time course of interleukin-8 content in bilateral nasal secretions after unilateral nasal allergen challenge. The arrow indicates the time of the allergen challenge. The dashed line represents values obtained after control challenge on a separate day using the same protocol [logarithmic y-axis, median and percentiles (10, 25, 50, 75, 90), $n = 12$, $*P < 0.05$, Wilcoxon-test].

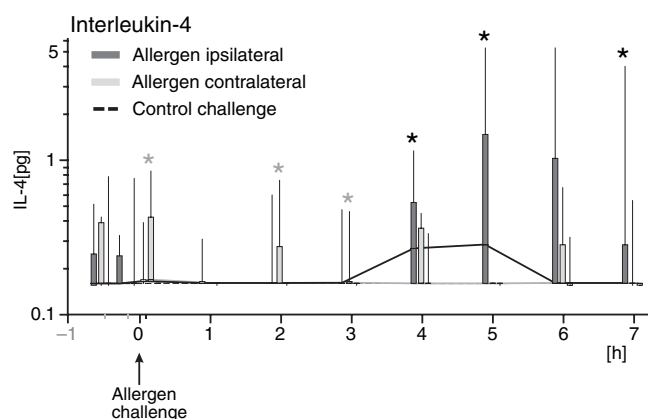


Figure 6. Time course of interleukin-4 content in bilateral nasal secretions after unilateral nasal allergen challenge. The arrow indicates the time of the allergen challenge. The dashed line represents values obtained after control challenge on a separate day using the same protocol [logarithmic y-axis, median and percentiles (10, 25, 50, 75, 90), $n = 12$, $*P < 0.05$, Wilcoxon-test].

phase but persists for hours after allergen contact by demonstrating the release of histamine and several cytokines into nasal secretions in the late phase. The disc method allowed the simultaneous measurement of physiological parameters, mediators and cytokines in nasal secretions. Other techniques like nasal lavage interfere with physiological parameters thus do not offer this advantage. Additionally, the disc method made it possible to investigate the mucosal reactions of both nostrils, enabling the differentiation between direct and indirect effects of the unilateral challenge.

In this and in previous studies (4, 6), an immediate increase of histamine could be demonstrated that can be assigned to a direct activation of mast cells after cross-linking of IgE by allergen. After a transient decrease histamine values rose again between 2 and 6 h after provocation. This time course is concordant with previous studies (1, 6). The increase of histamine in the late phase can be attributed to the activation of basophils that enter the nasal mucosa (6). The pathophysiological relevance of the high levels of histamine in the late phase remains unclear because they do not correlate with symptoms, secretions weights, or nasal flow.

The production of the proinflammatory cytokine IL-1 β has been demonstrated in inflammatory reactions of many different types. In the human nose it has been detected in acute viral rhinitis (7, 8) and chronic sinusitis (9). Our study showed a significant increase of IL-1 β in nasal secretions starting 2 h after challenge with peak values after 5 h. This observation contrasts with findings from Bachert and Ganzer (10) and Sim et al. (11) who also found elevations in the early phase. These discrepancies could be caused by the different methods of nasal allergen challenge and the collection of nasal secretions or by different time-points of the measurements in the early reaction, where we chose a much earlier time after challenge than the other investigators. Although the release of IL-1 β is not specific for allergic reactions, there are several observations that point to its relevance in allergic rhinitis. The reduction of IL-1 β in the nasal mucosa is one of the mechanisms attributed to the anti-inflammatory effects of topical steroids and immunotherapy in allergic rhinitis (12, 13). Furthermore, it could be demonstrated in a skin-model that the experimental treatment with a recombinant soluble IL-1Ra leads to an inhibition of the late phase reaction (14).

The IL-1Ra is a naturally occurring antagonist of IL-1 β . It binds with similar affinity to the same receptor as IL-1 β (IL-1R type 1), without activating it and can thus antagonize IL-1 β (15). Until now little is known about its occurrence in the human nose and the effects of allergen challenge on its release. We demonstrated that IL-1Ra is released into nasal secretions in concentrations that are 1000-times higher than IL-1 β . Allergen provocation leads to a modest increase. The nasal mucosa seems to have very effective means to confine the proinflammatory effects of IL-1 β in terms of time and space via the production of very high concentrations of IL-1Ra. Studies investigating the concentration of IL-1Ra in the course of the allergen season indicate that this anti-inflammatory capacity might be suppressed in active allergic rhinitis (16). As we were not able to demonstrate such an effect after experimental allergen provocation, we suspect that the regulation of IL-1Ra production in the nose has a time frame that lies apart from the one studied in our experiments.

Interleukin-8 is a member of the C-X-C family of chemokines. Its most important cellular sources in the

nasal mucosa are epithelial cells (17) and mast cells (18). We observed an increase of IL-8 in nasal secretions 5 min after allergen provocation and a second peak in the late phase. Similar results were found by Bachert and Ganzer (10) and Weido et al. (12) although the increase in the early phase did not reach statistical significance in these studies. This time course of IL-8 resembles the one of histamine and suggests a release of preformed IL-8 from mast cell granules. *In vitro* studies have indeed demonstrated that mast cells are capable of producing IL-8, storing it in granules, and releasing it after IgE-cross-linking (19). Nevertheless, some observations suggest that IL-8 does not play a central role in allergic rhinitis. Natural allergen exposure does not lead to significant changes in IL-8 in nasal secretions (16), and the blockage of IL-8 with specific antibodies reduces the chemotactic activity of allergen-induced nasal lavage fluid only marginally (20).

There is no doubt that IL-4 has a central role in the pathophysiology of allergic diseases. It is the archetypal Th2-cytokine and essential for the production of allergen-specific IgE. The IL-4 reinforces the development of a Th2-phenotype, antagonizes Th1 cells, and can induce a selective influx of eosinophils, basophils and T cells via the expression of the adhesion molecule vascular cell adhesion molecule 1 (VCAM-1) (21). Given these facts it is quite remarkable how little data can be found in the literature concerning the production of IL-4 in allergic rhinitis. A reason for this might lie in methodological problems attributed with its detection on the protein level: it is produced in small quantities, binds quickly to its receptors and underlies a prompt catabolism (22). The IL-4 mRNA in the human nose was first described by Durham et al. (23) in nasal biopsies after allergen challenge. Bradding et al. (24) was able to detect IL-4-positive cells immunohistochemically in the allergic nasal mucosa. Other groups were not able to detect IL-4 protein in nasal secretions after allergen challenge and in the allergen season (11, 25, 26). The reasons why we were able to measure IL-4 in nasal secretions might be: the very sensitive assay we used, the rapid collection and dilution of nasal secretions, and the localized challenge and collection. The kinetics of IL-4 release in our data show an increase hours after provocation with a peak at 5 h. The influx of eosinophils into the nasal mucosa follows a similar time course suggesting a causative relation through the expression of VCAM-1. IL-4 could be an attractive goal for new therapies as it has been demonstrated that one of the main effects of topical nasal steroids is the reduction of IL-4 mRNA expression (27). The potential clinical effectivity of an IL-4-directed therapy was proven for allergic asthma in studies with recombinant soluble IL-4 receptors (28).

In addition to determining the dynamics of the allergic reaction, the disc method allowed us to investigate indirect effects of nasal allergen challenge by measuring physiological changes and collecting secretions on the contralateral, unchallenged side of the nose. In previous

studies with this methodology we were able to show that the contralateral increase of nasal secretions is caused by a parasympathetically mediated nasonasal reflex (3, 4). Here, we demonstrate that unilateral nasal allergen challenge induces a bilateral release of histamine, which confirms previous studies (6), and also of cytokines, which has not been shown before. The amount of cytokines was much lower in the unchallenged nostril but the time course of their release was similar to the ipsilateral side. Two distinct mechanisms could explain the contralateral reaction: systemic effects of the nasal allergen challenge and neuronal reflexes. The relevance of systemic effects is supported by studies demonstrating that nasal allergen challenge leads to an influx of bone marrow precursor cells of the basophilic and eosinophilic lineage into the systemic circulation and from there into the airway mucosa (29). The fact that the influx of basophils is concomitant with the increase of histamine in the late phase supports this notion (6). Especially the early release of mediators and cytokines into nasal secretions of the unchallenged side suggests the operation of neural mechanisms that activate resident cells such as mast cells. The

most direct evidence for the relevance of neuronal mechanisms in allergic rhinitis stems from investigations where neurectomy of the vidian nerve was performed (30). This procedure leads to an interruption of the parasympathetic innervation of the nose and decreased the histamine release from mast cells after nasal allergen challenge. Further support for this assumption is related to the detection of neuropeptides in nasal secretions after nasal allergen challenge (31). They could induce the activation of resident or infiltrating cells and thus explain the contralateral release of mediators and cytokines (32, 33). We hypothesize that the contralateral increase of mediators and cytokines is caused by a combination of neuronal inflammation whose existence in the human nose has been demonstrated by studies from Togias and Sanico (34) and systemic effects of the allergen provocation.

Acknowledgment

This work was funded by the German research council (Deutsche Forschungsgemeinschaft: Wa 773/2-1).

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